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Transient Expression of GUS in Bombarded Embryogenic Longleaf, Loblolly, and Eastern White Pine

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Abstract

Embryogenic tissue cultures derived from immature zygotic embryos of longleaf, loblolly, and eastern white pine were maintained in culture for up to 2 years, then bombarded with gold particles coated with a gene construct containing the GUS reporter gene fused to an adenine methyltransferase promoter from an algal virus. Physiological expression of GUS was observed in cultures of all three pine species within 24 hours but not at 7 days. Expression of GUS activity was recorded in somatic embryonal heads of varied stages of development, suspensor cells and other cells of mixed ontogeny. Collective expression of GUS in small clusters of cells suggested inheritance of the reporter gene through early mitotic events. The presence of multiple discrete sites of GUS expression common in individual somatic embryos was indicative of densely associated multiple transformation events, and was enhanced by reducing the sample distance. This is a first report of transgene expression in longleaf and white pine.

Keywords: Biolistics, gene *gun, gene* promoter, GUS, *Pinus palustris* Mill., *P. strobus* L., *P. taeda* L.

Introduction

Microprojectile-mediated DNA transfer has been shown effective for a variety of woody angiosperms and gymnosperms. In the latter, the target tissues have included cotyledons (Stomp and others 1991, Sul and Korban 1994), mature pollen (Hay and others 1994, Li and others 1994), somatic embryogenic cultures (Clapham and others 1995, Duchesne and Charest 199 1, Bommineni and others 1994, Walter and others 1994), mature somatic embryos (Robertson and others 1992), vegetative buds and bud-derived calli (Aronen and others 1994), and xylem (Loopstra and others 1992). In such transformation attempts, expression of the newly introduced genetic loci has been most commonly transient for several possible reasons (Clapham and others 1995), although temporal expression has been lengthened (Clapham and others 1995), and even stabilized in some cases (Ellis and others 1993).

Our laboratories at Alabama A&M and Oklahoma State Universities cooperate on the development of methods for both vegetative propagation of southern pines and genetic manipulation for genotype improvement. The work reported here describes the results of microprojectile bombardment of embryogenic cultures of three southern pines, using an algal virus gene promoter as yet unreported for application with tree genetic manipulation. This is a first report of transgene expression in longleafand white pine embryogenic tissues.

Materials and Methods

Preparation of Embryogenic Tissues for Bombardment

Embryogenic cultures of white and longleaf pines were initiated and maintained for 2 years as described by Kaul (1995) and Nagmani and others (1993). Embryogenic cultures of loblolly pine were initiated from immature embryos collected in Idabel, OK, during mid-July 1995. Seeds excised from the cones were surface sterilized in 10 percent household bleach for 10 minutes, then rinsed. Megagametophytes removed from their developing seed coats were immersed for 5 minutes in 5 percent bleach, then rinsed and cultured on DCR medium (Becwar and others 1990) containing 2,4-dichlorophenoxyacetic acid (2,4-D) (12 μM [molar]) and benzylaminopurine (BAR) (4 μM) solidified using 0.2 percent Gehite. After 8 to 10 weeks, embryogenic calli were isolated and subcultured bi-weekly or tri-weekly for 4 months onto a freshly prepared medium of the same composition. At the end of that period

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(December 10, 1995), embryogenic cultures of all three species were transferred to a pH 5.8, 6.5 percent agar (Sigma Chem. Co., St. Louis, MO)-solidified Brown and Lawrence medium (Brown and Lawrence 1968) containing 2.5 percent sucrose, and modified to contain glutamine (10 mM) as the sole source of amino nitrogen. The medium was supplemented with 2,4-D (8 μM) and BAP (4 μM). All cultures were dark-incubated at 20-22 °C and subcultured every 3 weeks to freshly prepared medium.

Five to 7 days prior to bombardment, the embryogenic culture of each species was transferred to a freshly plated medium, to occupy an approximate circular area of 2- to 3-cm diameter in the center. Six replicate cultures were prepared for each species, of which one would be an unbombarded control.

Microprojectile Bombardment

Procedures used for microprojectile preparation and coating with DNA have been described by Heiser (1992). Each culture was bombarded once with 1 μg gold particles onto which had been precipitated "pAMTGUS25" (provided by A. Mitra, University of Nebraska, Lincoln, NE), containing *the Chlorella* virus adenine methyltransferase gene promoter. (Mitra and Higgins 1994) linked to the GUS reporter gene (Jefferson and others 1987). Twenty-five μg DNA was precipitated onto 3.6 mg gold particles. Initial bombardment employed a sample distance of approximately 6.3 cm. To increase particle pattern density, and thus greater potential transformation frequency, this was later reduced to 5 cm. All bombardments were carried out using a rupture-disc pressure of 1,100 psi. The apparatus employed for bombardment was the Biolistic™ particle delivery system PDS- 1000 (DuPont, Wilmington, DE). Following bombardment, petri dishes containing the cultures were incubated in the dark at 20 to 22 °C. Cultures were sampled at 24 hours, 48 hours, and 7 days. Sample size was approximately 0.5 cc. GUS activity was assayed as described (Jefferson and others 1987).

Results and Discussion

All bombarded embryogenic cultures expressed GUS activity at 24 hours, while the controls did not. Bombardment at a target distance of 6.3 cm produced approximately eight impacts/GUS-expression sites per 0.5 cc tissue, irrespective of species. Bombardment at 5 cm approximately doubled that effect, as measured at the maximum (40X) magnification provided by the binocular dissecting photomicroscope that was used to examine the microcentrifuge tube-content of bombarded tissue. However, greater resolution that was later provided by the compound microscope employed for photomicroscopy of transformant cells in thin layer, showed both multiple GUS-expressing sites on unit structures such as single embryos (fig. 1) and numerous isolated individual cells or small clumps (fig. 2) showing the same characteristic blue color. This observation was especially true of tissue bombarded at 5 cm.

Twenty-four and 48-hour assays showed GUS activity in single cells, as well as in small clusters of cells (fig. 2), apparently resulting from mitotic events following bombardment. Cells in both somatic embryo heads and suspensors showed GUS expression (fig. 1). Where cells were densely packed in the former, stain appeared either to have diffused into adjacent cells or to have reacted with substrate that had diffused from the specifically bombarded/GUS-expressing cell. This phenomenon was also reported for bombarded pine cotyledons (Stomp and others 199 1). The promoter employed here is a 85 l-base-pair upstream region from an algal virus methyltransferase gene, heretofore shown effective for expression in transformants of a small number of both monocots and dicots (Mitra and others 1994). No applications of this promoter to tree transformation have been reported. And, although our assays showed no GUS activity after 7 days, such a temporal loss of expression might be reduced through the use of certain medium osmotica or of different promoters (Martinussen and others 1994, Clapham and others 1995). Our work with somatic embryogenesis and genetic transformation of embryogenic tissues is continuing.

Acknowledgments

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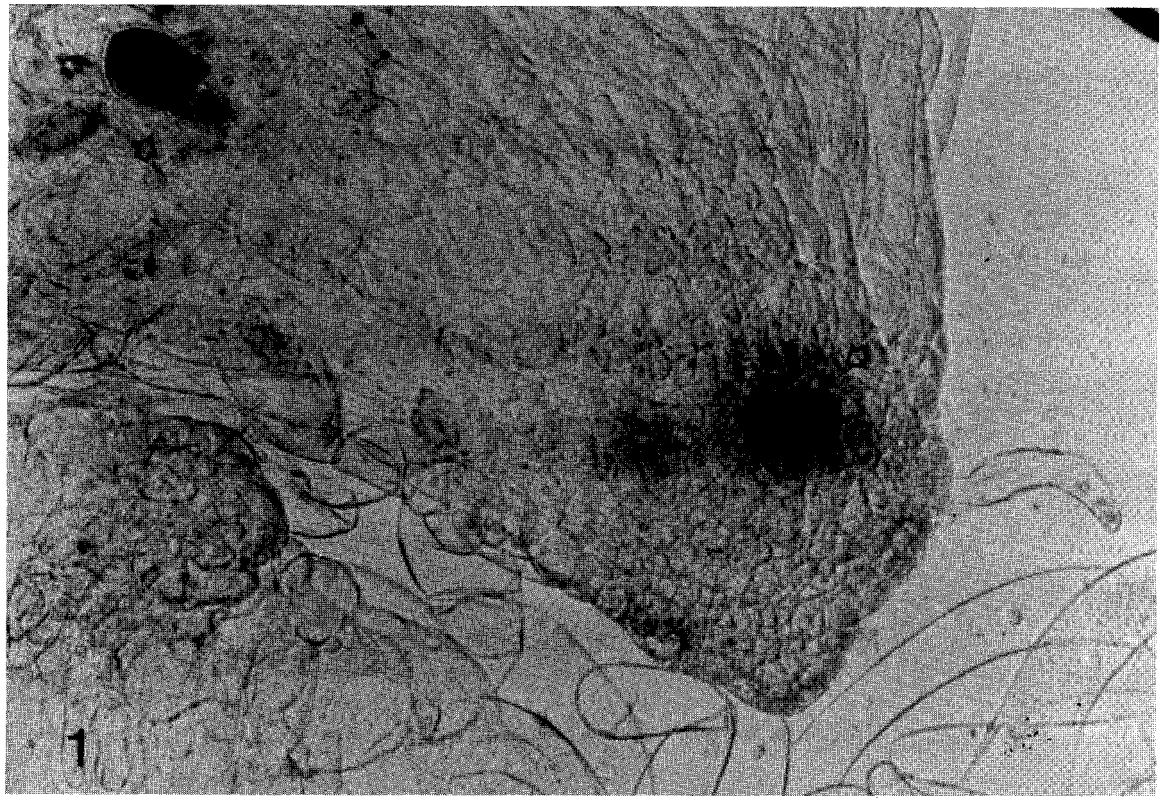

Figure 1—Longleaf pine somatic embryo, 48 hours after bombardment with the GUS reporter gene fused to the ***Chlorella*** *algal* virus promoter. Arrows indicate two sites expressing GUS. **600X**

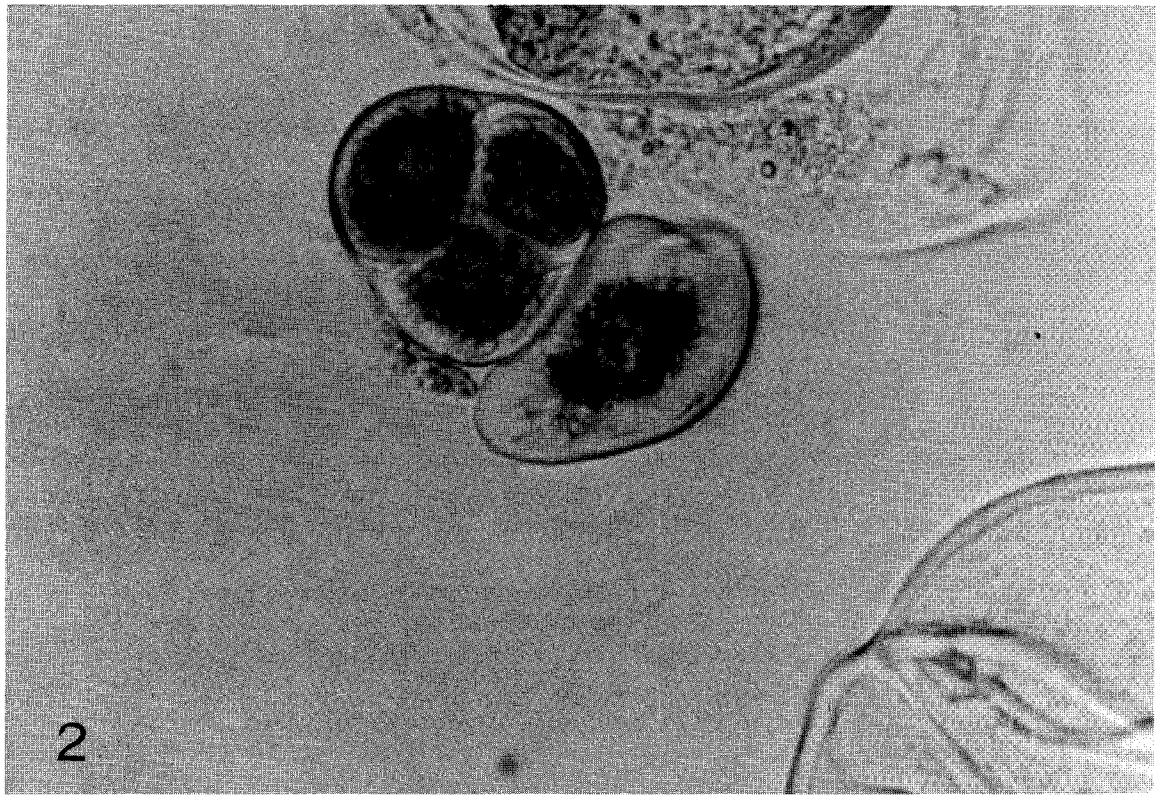

Figure 2-A **cluster** of four embryogenic cells of **eastern** white pine expressing GUS activity 48 hours **after** bombardment. At least three cells appear to have had a common progenitor. 1250X

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